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Metabolic dysfunction drives a mechanistically distinct pro-inflammatory phenotype in adipose tissue macrophages

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Abstract

Adipose tissue macrophage (ATM)-driven inflammation plays a key role in insulin resistance; however, factors activating ATMs are poorly understood. Using a proteomics approach, we show that markers of classical activation are absent on ATMs from obese humans, but readily detectable on airway macrophages of patients with cystic fibrosis, a disease of chronic bacterial infection. Moreover, treating macrophages with glucose, insulin, and palmitate – conditions characteristic of the metabolic syndrome – produces a ‘metabolically-activated’ phenotype distinct from classical

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AUTHOR CONTRIBUTIONS:

Conceived and designed the experiments: LB, MK, BRC, KH, SH. Performed the experiments: BRC, KH, DH, EP, KQS, VM, JNK. Recruited and coordinated patients undergoing abdominoplasty and bariatric surgery: IL, PSB, RWL, MC. Performed abdominal surgeries and collected adipose tissue samples: PSB, RWL, MC. Critically reviewed the manuscript: All authors. Wrote the manuscript: LB.

activation. Markers of metabolic activation are expressed by pro-inflammatory ATMs in obese humans/mice and are positively correlated with adiposity. Metabolic activation is driven by independent pro- and anti-inflammatory pathways, which regulate balance between cytokine production and lipid metabolism. We identify PPAR γ and p62/SQSTM1 as two key proteins that promote lipid metabolism and limit inflammation in metabolically-activated macrophages. Collectively, our data provide important mechanistic insights into pathways that drive the metabolic disease-specific phenotype of macrophages.

INTRODUCTION

Macrophages accumulate in adipose tissue of obese mice and humans (Weisberg et al., 2003; Xu et al., 2003) and are key contributors to inflammation and obesity-induced insulin resistance (Chawla et al., 2011; Lumeng and Saltiel, 2011; Olefsky and Glass, 2010; Wellen and Hotamisligil, 2005). The evidence implicating adipose tissue macrophage (ATM) inflammation in potentiating insulin resistance is substantial. Indeed, ablation of pro-inflammatory (CD11c+) ATMs using a diphtheria toxin system led to a rapid improvements in insulin sensitivity and glucose tolerance, associated with marked decreases in local and systemic inflammation in obese mice (Patsouris et al., 2008). Moreover, targeting pathways that mediate inflammation in the macrophage revealed significant roles for TLR4, JNK, and IKK β in potentiating ATM inflammation and insulin resistance in mice (Arkan et al., 2005; Han et al., 2013; Saberi et al., 2009). Anti-inflammatory effects may also help explain the insulin-sensitizing action of thiazolidinediones (TZDs). Indeed, myeloid-specific deletion of PPAR γ , the molecular target of TZDs, exacerbated macrophage inflammation and insulin resistance (Odegaard et al., 2007).

Macrophages are heterogeneous, and based on patterns of gene expression and function they have been classified as classically (M1) or alternatively (M2) activated (Gordon and Taylor, 2005). The M1 phenotype is promoted by Th1 mediators such as LPS and IFN γ and is characterized by the overproduction of pro-inflammatory cytokines. In contrast, Th2 mediators (eg. IL-4) drive the M2 phenotype, which activates expression of immunosuppressive factors and peroxisome proliferator-activated receptor gamma (PPAR γ) that promote tissue remodeling and helps resolve inflammation (Odegaard et al., 2007). It has been proposed that during weight gain macrophages undergo a 'phenotypic switch' from an anti-inflammatory M2 phenotype to a pro-inflammatory M1 state, a conversion that has been linked to the emergence of systemic insulin resistance (Lumeng et al., 2007).

Although it is clear that ATM activation is involved in regulating insulin sensitivity, the mechanisms that underlie transition to the pro-inflammatory ATM phenotype and corresponding signature of cell surface markers are poorly understood. For example, CD11c, the most commonly used marker of pro-inflammatory ATMs in mice and humans, is suppressed by the classical activation paradigm of LPS stimulation (Becker et al., 2012). More recently, Ferrante and colleagues provided evidence that ATMs in obese mice exhibit increased lipid metabolism and further suggested that increases in ATM number, rather than ATM activation, may be responsible for the low-grade inflammation observed in obesity (Xu et al., 2013). In addition, several studies have described a 'mixed' M1/M2 phenotype

for ATMs in obese mice and humans, suggesting that ATMs adopt more complex states *in vivo* (Sica and Mantovani, 2012; Zeyda et al., 2007). However the etiology of such mixed M1/M2 phenotypes are incompletely understood.

Here we combine proteomic, gene expression, and flow cytometric analyses of human and murine macrophages; studies of cells from obese, non-obese, and cystic fibrosis patients; and murine knockout studies to characterize pro-inflammatory ATMs, and to identify the molecular mechanisms that produce them. Our findings demonstrate that treating macrophages with mixtures of glucose, insulin, and palmitate (i.e., ‘metabolic activation’) produces a complex macrophage phenotype that is mechanistically distinct from classical activation, suggesting that metabolic disease-specific pathways drive macrophage inflammation via mechanisms that are different from those operative during infection.

RESULTS

Plasma membrane proteomics identifies markers of M1 macrophages

The lack of specific markers for human macrophage subsets is a major hurdle to understanding ATM inflammation in obese and diabetic patients (Geissmann et al., 2010). We therefore used a plasma membrane (PM) proteomics approach to define protein expression patterns diagnostic of pro-inflammatory human macrophages. Initial studies focused on a widely used *in vitro* system that included classically activated (M1, pro-inflammatory), alternatively activated (M2, anti-inflammatory), and un-stimulated (M0) macrophages generated from human peripheral blood monocytes (Mn) in the presence of M-CSF (MM0, MM1, MM2) or GM-CSF (GM0, GM1, GM2) (Fig. 1A). Classical and alternative activation were confirmed by measuring expression of genes associated with the M1 and M2 phenotypes (Fig. S1).

PM-associated proteins were biotinylated, affinity-isolated, and analyzed by liquid chromatography-electrospray ionization-tandem mass spectrometry. This approach identified 349 proteins with high confidence (Table S1). Gene ontology analysis of those proteins validated our analytical strategy by revealing strong enrichments in functional annotations consistent with the PM compartment of macrophages (Fig. S1).

Protein levels were quantified by spectral counting (Liu et al., 2004), a measure of relative protein abundance, which were closely correlated with flow cytometry data (Fig. S1). Markers of classically activated macrophages were selected by identifying proteins *consistently* up- (or down-) regulated by both the *t*-test and *G*-test (Becker et al., 2010) in M1 cells relative to M2 and M0 cells. We identified 52 significantly regulated PM proteins in M1 macrophages differentiated by M-CSF or GM-CSF treatment (Fig. 1B, Table S1). We further required that such markers were reproduced in macrophages differentiated in the presence of M-CSF and GM-CSF, as we thought this would increase their utility as markers in diverse environments *in vivo*. Based on these stringent criteria, we identified 13 membrane proteins as putative cell surface markers of M1 macrophages (Fig. 1B–C, Table S1).

We previously used a similar plasma membrane proteomics approach to define cell surface markers of M1 macrophages in mice (Becker et al., 2012). Interestingly, only 6 of the M1 markers identified in human macrophages overlapped with those found in classically activated bone marrow-derived murine macrophages (BMDMs). To promote continuity between human and mouse studies, our subsequent analyses focused on three such conserved markers (CD274, CD38, and CD319). Flow cytometric measurements validated the ability of CD274, CD38, and CD319 to reliably identify classically activated macrophages *ex vivo* using both human and murine macrophages (Fig. 1D, Fig. S1). Importantly, the induction of CD38 and CD319 on the cell surface of M1 cells was not attenuated by simultaneously exposing macrophages to M1 and M2 stimuli (Fig. 1E), suggesting that these markers can report exposure to classical stimuli even in the context of a ‘mixed’ macrophage phenotype, which is likely to be present in more complex conditions *in vivo* (Mosser and Edwards, 2008).

To validate the ability of these markers to identify macrophages inflamed by bacterial stimuli *in vivo*, we tested the expression of CD38, CD319, and CD274 on airway macrophages from infected patients with cystic fibrosis (CF), a disease characterized by heightened inflammatory responses in the airways due to chronic respiratory tract infection with pathogenic bacteria like *Pseudomonas aeruginosa* (Rowe et al., 2005). Flow cytometric analysis detected strong expression levels for all 3 markers, in all CF patients examined (Fig. 1F, Fig. S2). Thus, our proteomics approach successfully identified macrophage markers expressed in an *in vivo* inflammatory condition caused by chronic bacterial infection.

ATMs from obese humans and mice do not express M1 markers

During obesity, macrophages have been proposed to undergo a ‘phenotypic switch’ from an anti-inflammatory M2 phenotype to a pro-inflammatory M1 state (Lumeng et al., 2007). To determine if the M1 markers discovered by proteomics and validated in patients with CF could identify pro-inflammatory ATMs, we studied macrophages from omental and subcutaneous adipose tissue obtained from obese ($BMI > 30 \text{ kg/m}^2$) subjects undergoing bariatric surgery. For comparative purposes, we also investigated ATMs in subcutaneous adipose tissue obtained from non-obese ($BMI < 30 \text{ kg/m}^2$) subjects undergoing abdominoplasty. Although pro-inflammatory cytokine expression was elevated in both omental and subcutaneous adipose tissue from obese subjects (Fig. 2A), cell surface markers for M1 activation (CD38, CD274, CD319) were either not present or only very weakly expressed by ATMs in either of the adipose tissue depots examined (Fig. 2B–C, Fig. S3).

We also tested the ability of M1 markers to identify pro-inflammatory ATMs in mice. To this end, we used a mouse model for diet-induced obesity and insulin resistance (DIO), where male C57BL/6 mice were placed on low-fat or high-fat diets for 16 weeks. Analysis of ATMs from lean and obese mice recapitulated the findings obtained in human subjects. Thus, ATMs from obese mice were inflamed relative to their lean counterparts (Fig. 2D), but did not overexpress CD319, CD274, (Fig. 2E), or CD40 (Fig. 2E), a previously characterized M1 marker in mice (Becker et al., 2012). Importantly, treating ATMs (purified from obese mice) with LPS/IFN γ *in vitro* induced expression of CD319, CD274,

and CD40, suggesting that ATMs are capable of adopting an M1 phenotype given the appropriate environment (Fig. 2F). Collectively, these findings suggest that distinct surface markers report macrophage inflammation in a setting of bacterial infection and metabolic dysfunction.

'Metabolically' and classically activated macrophages express distinct cell surface proteins

The lack of M1 cell surface marker expression by pro-inflammatory ATMs in obese humans and mice suggested that distinct pathways might promote macrophage inflammation in the context of obesity, a hypothesis that has been proposed by Hotamisligil and colleagues (Calay and Hotamisligil, 2013; Hotamisligil and Erbay, 2008). In the context of metabolic disease, macrophage inflammation may be regulated by molecules such as glucose, insulin, and palmitate, which are elevated in patients with metabolic disease (Despres and Lemieux, 2006). Thus, we hypothesized that exposure to high levels of glucose, insulin, and palmitate ('metabolic activation'; MMe) may produce a unique pro-inflammatory macrophage phenotype.

Classical and metabolic activation (Fig. 3A) both induced pro-inflammatory cytokine expression (Fig. 3B). However, MMe macrophages failed to express cell surface markers diagnostic of classically activated macrophages including CD38, CD319, and CD274 (Fig. 3C), a finding that resembled our findings for pro-inflammatory ATMs isolated from obese humans and mice (Figs. 2B–C). To identify diagnostic cell surface markers of metabolic activation we performed membrane proteomics of MM0, MM1, and MMe macrophages. Proteomics analysis of metabolically and classically activated macrophages revealed remarkably distinct cell surface protein expression patterns (<5% overlap; Fig. 3D, Table S2). For example, cell surface proteins specifically over-expressed by MMe macrophages included ABCA1, CD36, and PLIN2 (Fig. 3E); proteins involved in lipid metabolism that have been associated with M2 macrophages. However, the M2 markers CD163, CD206, TFRC, and TGFBI (Becker et al., 2012; Martinez et al., 2006) were suppressed or not induced in MMe macrophages (Fig. 3F), indicating that metabolic and alternative activation programs are distinct. In addition, proteomics analysis confirmed that MMe macrophages did not express markers of classical activation (Table S2).

Adipose tissue media promotes 'metabolic activation' of macrophages

We considered the possibility that experimental exposure to mixtures of glucose, insulin, and palmitate may inadequately model *in vivo* ATM conditioning. To explore this, we treated human monocyte-derived macrophages with media conditioned by visceral adipose tissue from obese humans or mice, and found that *IL1 β* , *TNF α* , *ABCA1*, and *CD36* expression were all induced (Fig. 4A–B). In sharp contrast, treatment with sputum from a CF patient enhanced *IL1 β* and *TNF α* expression, and surface levels of CD274 and CD38, but failed to induce *CD36* and *PLIN2* in human macrophages (Fig. 4A). Thus, treating naïve macrophages with disease-specific biological fluids (adipose media vs. CF sputum) reproduces the distinct surface marker expression observed in airway macrophages and ATMs *in vivo* (Fig. 2,4).

Because adipocytes undergo lipolysis during culture and preferentially release palmitate into the medium, we hypothesized that palmitate was the major driver of metabolic activation. To test this hypothesis, we compared MMe macrophages with MM0 macrophages treated with glucose, insulin, or palmitate in isolation. Treatment with palmitate was sufficient to drive *PLIN2* and *IL1 β* expression, suggesting that it may be the main driver of metabolic activation (Fig. S4). We further confirmed that palmitate levels at the lower limit in type 2 diabetic patients (0.1mM) (Reaven et al., 1988) could stimulate the MMe phenotype as well (Fig. S4).

ATMs of obese humans and mice display evidence of ‘metabolic activation’

We investigated if markers of metabolic activation were present on ATMs *in vivo* by quantifying ATM cell surface ABCA1 and CD36 levels in omental and subcutaneous adipose tissue from obese subjects undergoing bariatric surgery. As before, subcutaneous adipose tissue obtained from non-obese (BMI<30kg/m²) subjects undergoing abdominoplasty were used for comparative purposes. Cell surface CD36 and ABCA1 levels were elevated on ATMs from omental and subcutaneous adipose tissue in obese relative to non-obese subjects and relative to airway macrophages of CF patients (Fig. 4C, Fig. S3). Moreover, linear regression analysis revealed strong relationships between BMI and ATM CD36 levels ($R^2 = 0.73$, $p=0.0001$, $N=14$) or ABCA1 levels ($R^2 = 0.40$, $p=0.02$, $N=14$) in subcutaneous adipose tissue (Figs. 4D–E), suggesting that macrophage metabolic activation correlates with adiposity. In sharp contrast, such relationships were absent for markers of M1 macrophages assessed in the same patients (CD38, CD319, CD274; Figs. 4D–E).

Analysis of ATMs from obese mice produced similar findings. Indeed, we observed increases in the expression of pro-inflammatory cytokines (*Tnfa* and *Il1 β*) and MMe markers (*Plin2*, *Abca1*) in ATMs isolated from obese mice (Fig. 4F). Moreover, ATMs isolated from obese mice overexpressed 7 of 9 additional MMe markers identified in our proteomics analysis of human MMe macrophages (Fig. 4G). Collectively, these findings are consistent with the notion that palmitate released from adipocytes promotes macrophage metabolic activation *in vivo*.

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Pro-inflammatory signaling does not drive MMe surface marker expression

The fact that M1 and MMe macrophages differed markedly in surface marker expression was surprising given that palmitate is thought to mimic LPS action by binding to TLRs (Himes and Smith, 2010; Shi et al., 2006). This finding led us to hypothesize that different signaling pathways mediate the M1 and MMe phenotypes. To begin to test this hypothesis, we used bioinformatics to identify specific cell signaling pathways linked to cell surface proteins expressed by MM1 (but not MMe) macrophages.

LPS binding to TLR4 stimulates pro-inflammatory cytokine production through the NF κ B pathway and induces the type I interferon response via IRF7 (Beutler, 2004). Consistent with this idea, bioinformatics analysis suggested that cell surface proteins selectively induced by MM1 macrophages were associated with the type I interferon response ($p=10^{-30}$;

Fig. 5A). To confirm the role of the type I interferon pathway in regulating M1 surface markers, we performed two experiments that engaged the NF κ B pathway without a type I interferon response. First, we compared CD38, CD274, and CD319 expression in MM1 cells made from wild type and type I interferon pathway deficient (*Ifnar*^{-/-}) mice (Muller et al., 1994). Ablation of *Ifnar1* significantly lowered cell surface CD38, CD274, and CD319 expression in M1 macrophages (Fig. 5B). Second, we stimulated human macrophages with the TLR2 agonist Pam2CSK4. Pam2CSK4 induced *IL1 β* , *TNF α* , and *IL6* expression to levels similar to MM1 macrophages (Fig. 5C) but did not elicit the type I interferon response (Toshchakov et al., 2002) (Fig. 5D) or CD38, CD274, and CD319 expression (Fig. 5E). In addition, Pam2CSK4 treatment did not elevate ABCA1, CD36, and PLIN2, suggesting that this agonist does not stimulate metabolic activation (Fig. 5F).

Given that the type I interferon pathway controls CD38, CD274, and CD319 expression in M1 macrophages, and that MMe cells express different cell surface proteins, we postulated that the type I interferon response would not be activated in these cells. As expected, neither human nor murine MMe macrophages exhibited *IRF7* gene expression and IKK ϵ phosphorylation (Fig. 5G–H, Fig. S5), two key drivers of the type I interferon response (Honda et al., 2005). Pro-inflammatory ATMs from obese mice also did not exhibit elevated IKK ϵ phosphorylation or *IRF7* gene expression relative to lean mice (Fig. 5I–J).

Since MMe macrophages fail to engage the type I interferon response, and activation of this pathway is a key feature of TLR4 activation, we investigated whether surface markers of MMe macrophages were dependent upon the presence of TLRs. Ablation of *Tlr2* or *Tlr4*, which mediate the pro-inflammatory effects of palmitate on macrophages (Himes and Smith, 2010; Shi et al., 2006), attenuated *Il1 β* expression but did not alter *Plin2* expression in MMe macrophages (Fig. 6A). Collectively, these studies demonstrate that unlike MM1 macrophages, cell surface markers of MMe macrophages are independent of the type I interferon pathway and TLR activation.

p62 and PPAR γ promote MMe surface marker expression

What molecular mechanisms drive cell surface marker expression in MMe macrophages? We used two approaches to exploit our proteomics data to identify signaling pathways controlling cell surface marker expression in MMe macrophages.

First, we used bioinformatics analysis and found that surface proteins selectively induced in MMe macrophages were involved in lipid metabolism and regulated by PPAR γ (Fig. 6B, $p=10^{-7}$). Consistent with the informatics data, chromatin immunoprecipitation (ChIP) studies demonstrated increased binding of PPAR γ to the promoters of *PLIN2*, *ABCA1*, and *CD36* in human and murine MMe macrophages (Fig. 6C, Fig. S6) and PPAR γ promoter binding correlated with histone H3K4 trimethylation (H3K4m3), an epigenetic mark for open chromatin (Fig. 6C). Furthermore, a PPAR γ antagonist (T0070907; 1 μ M) reduced *ABCA1*, *PLIN2* and *CD36* expression in MMe macrophages (Fig. S6).

Second, we searched the membrane proteome for proteins with well-established roles in cell signaling that were selectively induced in MMe macrophages. This analysis identified a strong and selective induction of sequestome-1 (p62) (Fig. 6D). Moreover, p62 was

significantly elevated in ATMs from obese relative to lean mice (Fig. 6D). p62 is a scaffolding protein that regulates a variety of cell signaling cascades (Komatsu et al., 2007). Previous studies suggest that p62 regulates adipocyte lipid metabolism and adiposity in mice (Muller et al., 2013; Rodriguez et al., 2006). To determine if p62 regulates the MMe phenotype, we compared macrophages from wild-type and p62-deficient (*p62*^{-/-}) mice and found *Plin2*, *Abca1*, and *Cd36* expression was reduced in *p62*^{-/-} cells (Fig. 6E). Thus, p62 and PPAR γ regulate expression of CD36, ABCA1, and PLIN2 in MMe macrophages.

p62 and PPAR γ limit inflammation during 'metabolic activation'

The induction of PPAR γ in pro-inflammatory MMe macrophages seemed surprising given that saturated FFAs are poor PPAR γ ligands (Wahli and Michalik, 2012) and that PPAR γ exerts anti-inflammatory effects (Odegaard et al., 2007). We examined the effect of PPAR γ on the inflammatory phenotype of MMe macrophages by treating cells with the PPAR γ antagonist T0070907. Consistent with an anti-inflammatory role of PPAR γ (Odegaard et al., 2007), blocking PPAR γ with T0070907 increased expression of *IL1 β* in MMe cells (Fig. S6). The p62 induction identified in MMe macrophages (Fig. 4D) also had an anti-inflammatory effect, as *p62*^{-/-} macrophages markedly over-produced *Tnfa* and *Il1 β* in response to metabolic, but not classical activation (Fig. 6E). These data indicate that p62 and PPAR γ attenuate pro-inflammatory cytokine production during metabolic activation, which may in part explain why the sterile inflammation associated with metabolic disease is often of lower intensity than that induced by bacterial infection.

Palmitate uptake induces P62 accumulation by inhibiting autophagy

What mechanism induces p62 during metabolic activation? Whereas p62 is normally degraded by the autophagolysosome, it accumulates under conditions of failed autophagy (Komatsu et al., 2007). Consistent with a blockade in autophagy in MMe macrophages, p62 protein accumulated in a time dependent fashion (Fig. 6F) and its accumulation could not be explained by increased transcription (Fig. 6G). Similarly, LC3-II protein, another marker of failed autophagy (Shpilka et al., 2011), also accumulated in MMe macrophages (Fig. 6F). Moreover, treatment with chloroquine (an inhibitor of autophagy) induced LC3-II accumulation in MM0 macrophages, but not in MMe macrophages (Fig. S7).

How could metabolic activation inhibit autophagy? One possibility is that this inhibition of autophagy is linked to the pro-inflammatory state. Arguing against this possibility, MM1 macrophages did not accumulate p62 (Fig. 6D), and inactivation of *Tlr2* or *Tlr4* in MMe macrophages abrogated pro-inflammatory cytokine expression (Fig. 6A), but had minimal effect on p62 (Fig. 6H).

In addition to binding to TLRs, palmitate is also internalized by macrophages (Fig. 6I), and the kinetics of palmitate internalization and p62 accumulation in human macrophages was well correlated (Fig. 6I). Furthermore, experiments in which we increased the amount of palmitate ingested per cell by varying the palmitate: MMe macrophage ratio in culture markedly increased p62 accumulation (Fig. 6J). Interestingly, ablation of *Cd36* had no effect on palmitate uptake by BMDMs *in vitro* (Fig. S4), and previous studies demonstrated normal palmitate uptake in *Cd36*^{-/-} ATMs (Nicholls et al., 2011). Collectively, these

findings suggest that CD36-independent palmitate uptake has a greater effect in suppressing autophagy and inducing p62 accumulation than palmitate recognition by cell surface TLRs.

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DISCUSSION

ATM inflammation plays a key role in obesity-associated insulin resistance and type 2 diabetes (Chawla et al., 2011; Lumeng and Saltiel, 2011; Olefsky and Glass, 2010; Wellen and Hotamisligil, 2005). A common observation reported in studying macrophages in obese mice and humans, is that ATMs overexpress pro-inflammatory cytokines via signaling pathways that are operative in M1 macrophages, yet they cannot be identified when using cell surface markers of classical activation.

One possible explanation for these discordant findings is that currently available surface markers for M1 macrophages, particularly in humans, may not be as specific as previously anticipated (Geissmann et al., 2010). To circumvent this problem, we used a proteomics approach to define a set of cell surface proteins that *i*) report classical activation in human monocyte-derived macrophages differentiated in the presence of M-CSF or GM-CSF (two well-established paradigms for generating macrophages *in vivo*) *ii*) are maintained in macrophages exposed to mixed M1/M2 stimuli, *iii*) are conserved between human and mouse macrophages, and *iv*) are validated *in vivo* in a setting of chronic bacterial infection (CF patients).

Our findings show that markers identified by such stringent approaches still fail to characterize pro-inflammatory ATMs from obese subjects, both in omental and subcutaneous adipose depots. Similar findings were obtained in our analyses of ATMs isolated from obese mice in a DIO model. Collectively, these data provide strong evidence that pro-inflammatory ATMs do not adopt the classically activated M1 phenotype.

Our demonstration that cell surface markers of classical activation are primarily regulated by the type I interferon response may have important implications for how pro-inflammatory ATM phenotypes (and macrophage phenotypes in other diseases) are conceptualized *in vivo*. Thus, dissociation of mechanisms that control cell surface marker expression (how we detect macrophages) from pathways that control cytokine production (how we detect inflammation) enables these processes to be regulated independently. In a setting of classical activation, both pathways are co-regulated by LPS (Beutler, 2004).

However, our data show that metabolic stimuli promote inflammation without activating the type I interferon response, a finding that is consistent with the inability of M1 markers to identify M1 macrophages *in vitro* and pro-inflammatory ATMs *in vivo*. Moreover, this concept may not be restricted to settings of sterile inflammation as bacterial products that are primarily recognized by TLR2 also produce inflammation without activating the type I interferon response (Toshchakov et al., 2002), an observation that we confirmed experimentally. Based on these findings, it is likely that the repertoire of cell signaling pathways induced by a particular pro-inflammatory stimulus, and not necessarily the extent

of inflammation, determines the cell surface markers that will ultimately be useful for interrogating that cell type *in vivo*.

Thus, we propose that cell surface markers of macrophages be used not to identify ‘cellular phenotypes’, but rather as robust indicators of specific cell signaling pathways that are activated in a given microenvironment. Our approach for using disease-specific conditions to define novel surface markers and delineate mechanisms driving their expression can therefore provide insights into the signaling pathways engaged and the functional properties of the macrophages. In this respect, proteins regulated by the type I interferon response, which identify M1 macrophages, modulate host anti-viral and anti-bacterial responses (Honda et al., 2005). On the other hand, metabolic dysfunction and nutrient excess impose unique challenges for ATMs. Insulin resistance in the adipocyte leads to a continuous and excessive exposure of ATMs to FFA, particularly saturated FFA such as palmitate, which our data suggest plays a key role in triggering metabolic activation of macrophages. Thus, the induction of proteins involved in lipid metabolism, which delineate MMe macrophages, may enable these macrophages to appropriately buffer their environment from excessive lipids while maintaining cell health. Importantly, saturated FFAs up-regulate pro-inflammatory cytokine expression in ATMs and fail to induce the M2 markers CD163, CD206, TFRC, and TGFBI (Becker et al., 2012; Martinez et al., 2006), properties that clearly distinguish these cells from M2 macrophages, which also induce lipid metabolism through PPAR γ activation (Odegaard et al., 2007).

On a molecular level, the metabolically activated macrophage phenotype is mediated by at least two independent mechanisms: a pathway involving palmitate binding to cell surface TLRs that drives pro-inflammatory cytokine production, and a pathway mediated by palmitate internalization which activates p62 and PPAR γ , thereby promoting lipid metabolism and limiting inflammation (Fig. 7). The balance between these two processes determines the overall response of macrophages to metabolic dysfunction (ie. pro- or anti-inflammatory), and can produce complex macrophage phenotypes spanning the spectrum between the ‘M1-like’ and ‘M2-like’ states. Indeed, our analyses demonstrate strong correlations between cell surface CD36 and ABCA1 levels on ATMs (markers often associated with M2 macrophages, (Martinez et al., 2006)) and metabolic dysfunction in obese humans and mice. Concordant with our findings, previous studies suggested the presence of mixed macrophage phenotypes in obese human subjects and mice (Sica and Mantovani, 2012; Zeyda et al., 2007).

Our model further predicts that excessive accumulation of palmitate within the macrophage would further attenuate or silence inflammation via p62 and PPAR γ activation, and promote a more ‘M2-like’ ATM phenotype characterized by increased lipid metabolism. Factors influencing the amount of FFAs ingested by macrophages include adipose tissue mass, adipocyte size, and extent of insulin resistance in the adipocyte, all of which would increase with the degree of metabolic dysfunction in patients with type 2 diabetes. In mice, we would expect that duration of high-fat feeding regimens and the mouse model of obesity used (*Lep^{ob/ob}* develop more severe metabolic dysfunction than DIO) to greatly influence the ATM phenotype observed. Consistent with these assumptions, previous studies demonstrated that obesity-induced shifts in ATMs from a ‘M2-like’ to a ‘M1-like’

phenotype (Lumeng et al., 2007) are reversed when mice are fed a high-fat diet for extended periods of time (Shaul et al., 2010). Similarly, recent work by Ferrante and colleagues showed that ATMs of obese *Lep^{ob/ob}* mice (obese BW = 50–70g) are not inflamed, but rather are characterized by increased lipid metabolism (Xu et al., 2013). In our analyses of more mildly obese and insulin resistant mice (DIO, obese BW = 30–35g) we observed up-regulated cytokine expression in ATMs. However, we also noted increases in genes involved in lipid metabolism suggesting that the antiinflammatory pathway is operative, but not yet dominant.

In summary, our findings demonstrate that metabolic dysfunction leads to a macrophage phenotype that is mechanistically distinct from classical activation, suggesting that disease-specific environments drive macrophage inflammation via different mechanisms. Importantly, our data do not imply that treating macrophages with adipose tissue or mixtures of glucose, insulin, and palmitate can holistically model the ATM phenotype since conditioning environments *in vivo* are more complex and dynamic. However, our data suggest that metabolic activation is a suitable model for conceptualizing ATM phenotype and function, as it reconciles the seemingly contradictory findings of elevated pro-inflammatory cytokine expression in a cell expressing markers of an ‘M2-like’ phenotype. Future gene knockout studies that specifically attenuate signaling pathways driving metabolic activation will be required to more carefully delineate its contribution to ATM inflammation and metabolic disease phenotypes *in vivo*.

EXPERIMENTAL PROCEDURES

Ethics

All human studies were approved by the Institutional Review Boards at the University of Washington (IRB#13293), Seattle Children’s Hospital (IRB#31279), and Fred Hutchinson Cancer Research Center (IRB#6874). Animal studies were approved by the Institutional Animal Care and Use Committee (ACUP#72209) at the University of Chicago.

Differentiation and activation of human monocyte-derived macrophages

Peripheral blood monocytes were isolated from healthy donors and differentiated to macrophages in the presence of M-CSF (125ng/mL) or GM-CSF (10ng/mL) as previously described (Martinez et al., 2006). Treatments and activations were performed for 24h as follows: MM1: LPS (100ng/mL) and IFN γ (20ng/mL), MM2: IL4 (20ng/mL), MMe: glucose (30mM) and insulin (10nM) and palmitate (0.4mM); human adipose tissue conditioned media (1/10 dilution); Pam2CSK4 (100ng/mL); Chloroquin (100mM). Resultant macrophage populations were subjected to analysis by flow cytometry, qRT-PCR, immunoblotting, and plasma membrane proteomics.

Plasma membrane proteomics

Macrophage plasma membrane proteins were isolated using a membrane-impermeable, biotinylation reagent (N-hydroxysulfosuccinimide-SS-biotin; Pierce) as previously described (Becker et al., 2012). Mass spectrometric analyses and statistical analyses of

proteomics data were performed essentially as previously described (Becker et al., 2010). Full details are provided in the online supplement.

Bioinformatics analysis

Functional analyses of the proteomics data were generated through the use of IPA (Ingenuity® Systems, www.ingenuity.com). Informatics data were analyzed by the hypergeometric test with Benjamini-Hochberg correction.

Subject recruitment (elective abdominal surgeries)

Human adipose tissue was obtained from anonymous donors undergoing abdominoplasty (N=7) at Seattle Plastic Surgery, or from study participants undergoing laparoscopic intraabdominal surgeries (N=7) at the Puget Sound Surgery Center in Edmonds, WA. Exclusion criteria included smoking; abuse of alcohol or other drugs; pregnancy; history of cardiovascular, autoimmune, or other chronic inflammatory disease; or current or recent use of anti-diabetic, anti-inflammatory, or steroid hormone drug.

Adipose tissue collection and processing

Human adipose tissue was collected by surgeons conducting abdominoplasty (subcutaneous only) or intraabdominal surgery (subcutaneous and omental) and processed within 10 minutes by study staff. Stromavascular cells were isolated as previously described (Hagman et al., 2012). In some instances, a 100mg piece of freshly harvested adipose tissue was cultured in 1mL media for 24h to obtain human adipose tissue conditioned media.

Subject recruitment (Cystic Fibrosis)

Spontaneously expectorated sputum was collected in clinic, or sent overnight on ice from the patient's home. Study participants were older than 18 years of age and had a confirmed diagnosis of CF. Exclusion criteria included significant hemoptysis within 2 weeks of collection or a prior lung transplant.

Sputum processing

To solubilize sputum, samples were mixed with an equal volume of 0.1% DTT in PBS and placed on ice for 30min with intermittent vortexing. Samples were passaged through sterile cotton gauze and filtered through 70 µm nylon gauze cell strainers. Cells were centrifuged at 300×g for 10 min at 4°C, and washed 4 times with 50ml RPMI to reduce debris. Cells were counted and assessed for viability using trypan blue exclusion. If contaminated with saliva, salivary components were aspirated from the sample prior to sputum processing.

Mouse studies

Male C57BL/6 mice were obtained from Jackson laboratories. BMDMs were isolated as previously described (Becker et al., 2012) and activated along the MM1 and MMe pathways using identical conditions as for the human macrophages. For DIO studies, mice were fed a low-fat (Harlan Tekland; 2918) or high-fat (Research Diets Inc.; D12451) diets for 16 weeks. Epididymal adipose tissue was collected, washed, subjected to collagenase digestion

to isolate stromavascular cells, and ATMs were purified using anti-CD11b coupled magnetic beads (Miltenyi).

Flow cytometry

Fluorochrome labeled cells were analyzed according to the workflows presented in Fig. S2, S3. Analyses were conducted using a Canto-II or LSRII flow cytometer (BD Biosciences) and data were analyzed using FlowJo software v.9.4.11. Protein levels were quantified by mean fluorescence intensity and normalized to isotype to facilitate comparison between patients.

Antibodies

Antibodies for flow cytometric measurements of ABCA1, CD1c, CD11c, CD14, CD15, CD16, CD36, CD38, CD45, CD274, and CD319 were purchased from BD Biosciences (San Jose, CA), Beckman Coulter (Danvers, MA), Novus Biologicals (Littleton, CO) BioLegend (San Diego, CA), eBioscience (San Diego, CA), or Miltenyi (Auburn, CA).

Chromatin Immunoprecipitation

ChIP experiments were performed as previously described (Mutskov et al., 2002) using specific primers (Table S3) designed based on previously published PPAR γ ChIP-Seq data (Mikkelsen et al., 2010) and the RXR-binding sites from the ENCODE project. Full details are provided in the online supplement.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Research highlights

- ❖ Stimuli associated with metabolic disease promote macrophage metabolic activation.
- ❖ Metabolic activation involves distinct mechanisms and surface markers.
- ❖ ATMs of obese humans/mice overexpress metabolic activation markers, not M1 markers.
- ❖ Markers of metabolically activated macrophages are positively correlated with BMI.

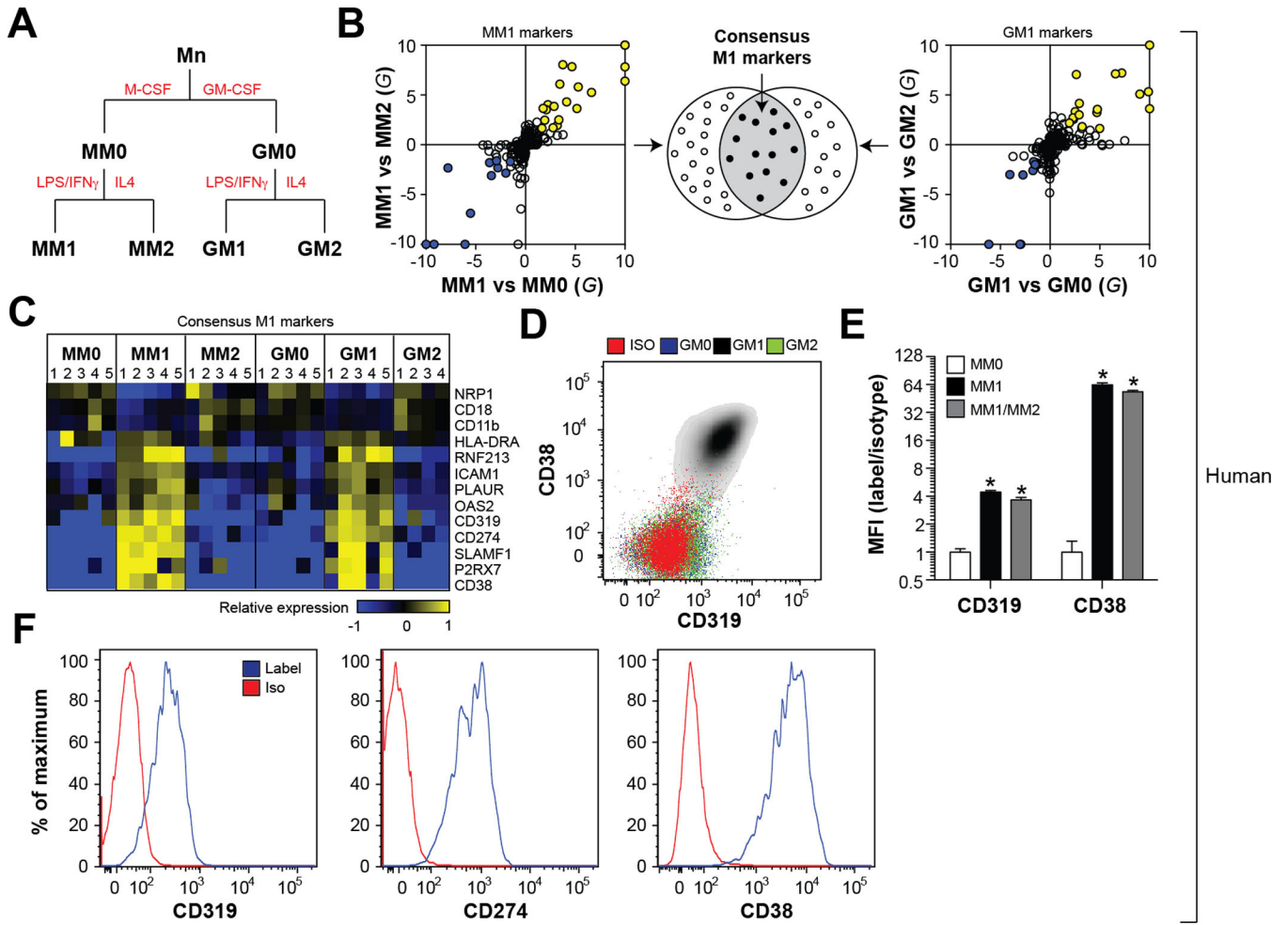


Fig. 1. Plasma membrane proteomics identifies markers of M1 macrophages

Panel A: Conditions for differentiating and activating human monocyte-derived macrophages. *Panel B:* Proteins consistently up- (or down-) regulated in M1 relative to M0 and M2 macrophages were identified using a combination of the *G*-test and *t*-test. *Panel C:* The consensus markers of M1 macrophages; heatmap depicts regulation of protein expression across all cells types (blue = down-regulated, yellow = up-regulated). *Panel D:* Validation of M1 markers by flow cytometry. *Panel E:* Cell surface levels of CD319 and CD38 in macrophages exposed to M1 or M1/M2 (LPS, IFN γ , and IL4); results are signal-to-noise ratio in mean fluorescence intensity (MFI). *Panel F:* Cell surface CD319, CD274, and CD38 levels in airway macrophages (defined as CD14⁺CD15⁻) of a CF patient. Where applicable, results are means and SEMs; *, denotes $p < 0.05$, *t*-test relative to MM0; N=6. See also Figs. S1, S2; Table S1.

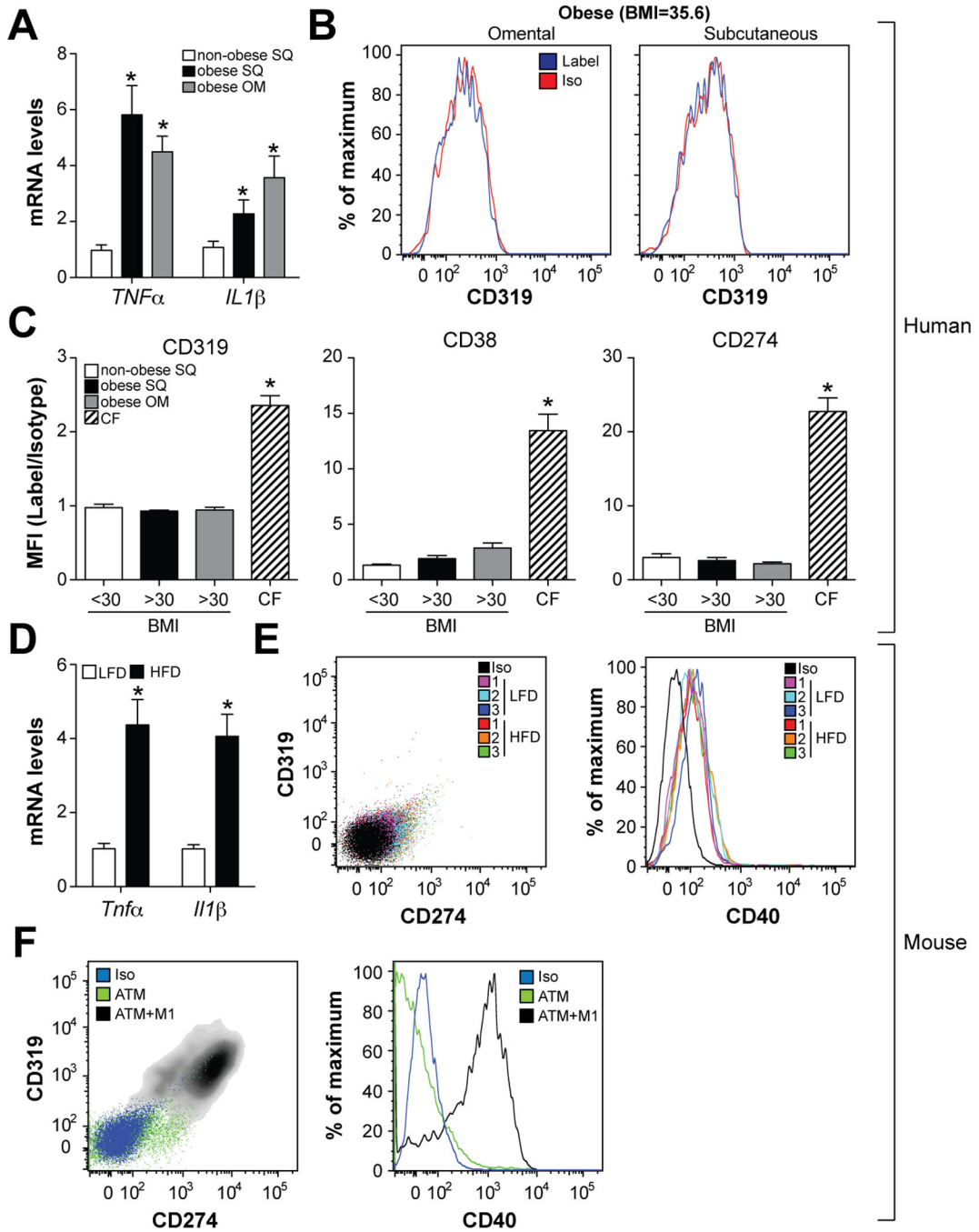


Fig. 2. ATMs of obese humans and mice are not classically activated

Panels A-C: Omental (OM) and subcutaneous (SQ) adipose tissue was collected from obese subjects (BMI>30kg/m²) undergoing bariatric surgery (N=7). For comparative purposes, subcutaneous adipose tissue was collected from non-obese subjects (BMI<30kg/m²) undergoing abdominoplasty (N=7). *Panel A:* Pro-inflammatory cytokine expression. *Panel B:* Cell surface CD319 levels in ATMs (defined as CD14⁺CD15⁻CD206⁺CD1c⁻) from a representative obese subject. *Panel C:* Cell surface levels of CD38, CD274, and CD319 in ATMs from non-obese and obese subjects and airway macrophages from CF patients;

results are signal-to-noise ratio in mean fluorescence intensity (MFI). *Panels D-F*: Male C57BL/6 mice were placed on a low-fat diet (LFD) or high-fat diet (HFD) for 16 weeks and ATMs from epididymal fat were isolated. *Panel D*: Pro-inflammatory cytokine expression. *Panel E*: ATM cell surface CD319, CD274, and CD40 levels. *Panel F*: ATM cell surface CD319, CD274, and CD40 levels in the presence and absence of M1 stimuli. Where applicable, results are means and SEMs; *, denotes $p < 0.05$, t -test relative to non-obese or LFD; N=4–7. See also Fig. S3.

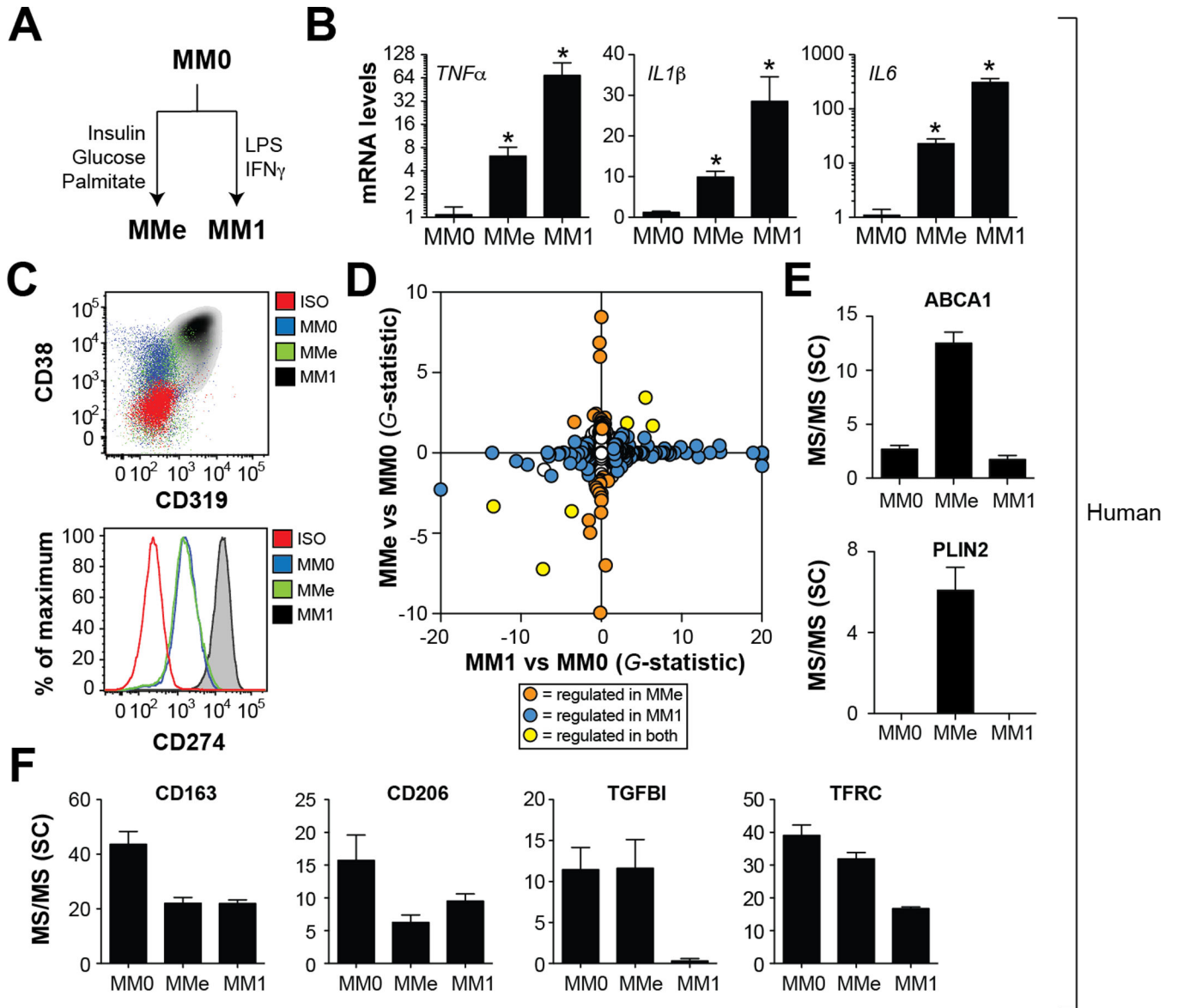


Fig. 3. Metabolically and classically activated macrophages are distinct
Panel A: Conditions for metabolic (MMe) or classical (MM1) activation of human monocyte-derived macrophages. *Panel B:* Pro-inflammatory cytokine expression. *Panel C:* Flow cytometric analysis of cell surface CD38, CD319, and CD274 levels in MM0, MM1, and MMe macrophages. ISO= isotype control. *Panel D:* Plasma membrane proteomics analysis of MM0, MM1, and MMe macrophages. *Panels E-F:* Quantification of cell surface proteins in MM0, MM1, and MMe macrophages by mass spectrometry (spectral count, SC). Where applicable, results are means and SEMs; *, denotes $p < 0.05$, t -test relative to MM0; N=6. See also Table S2.

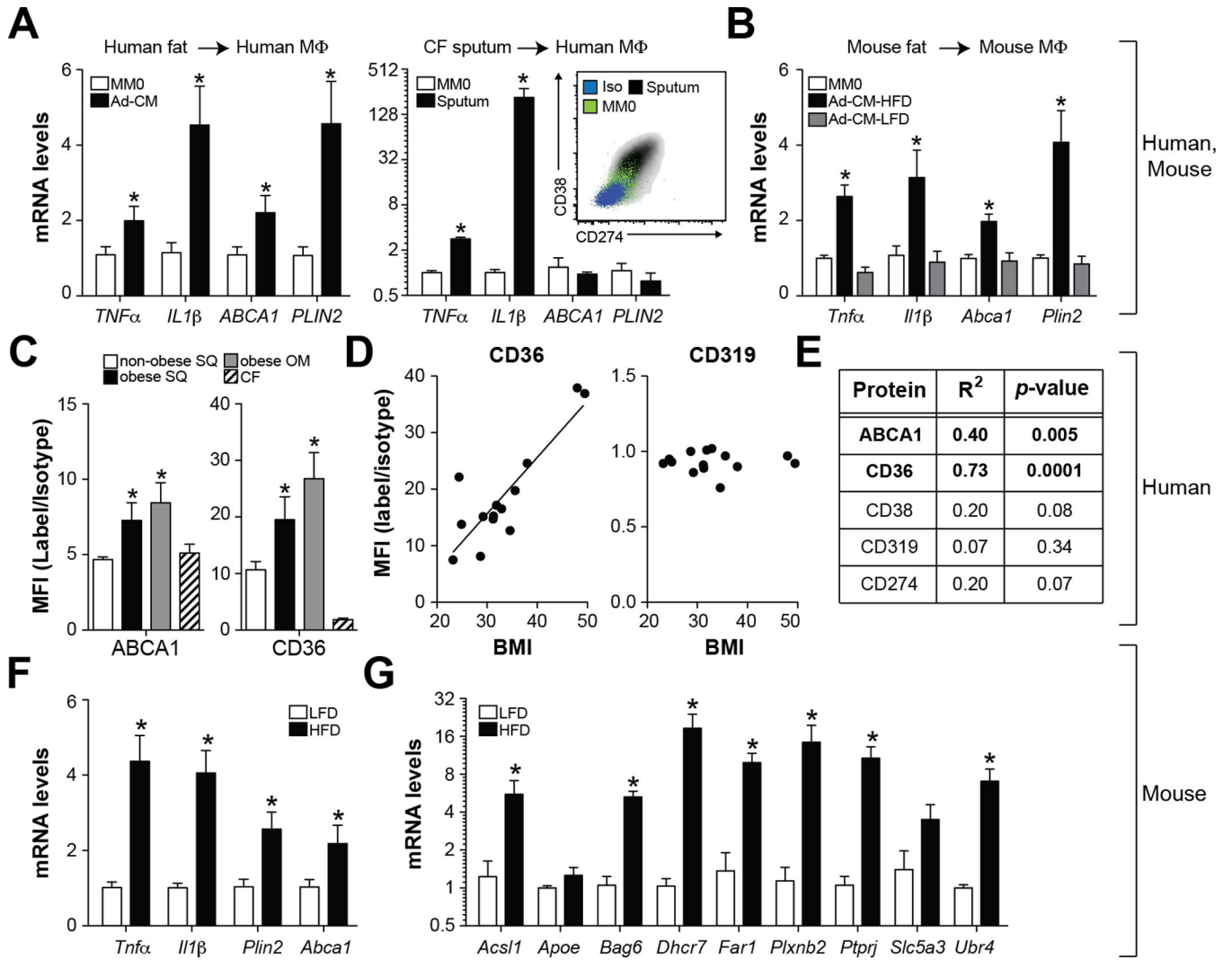
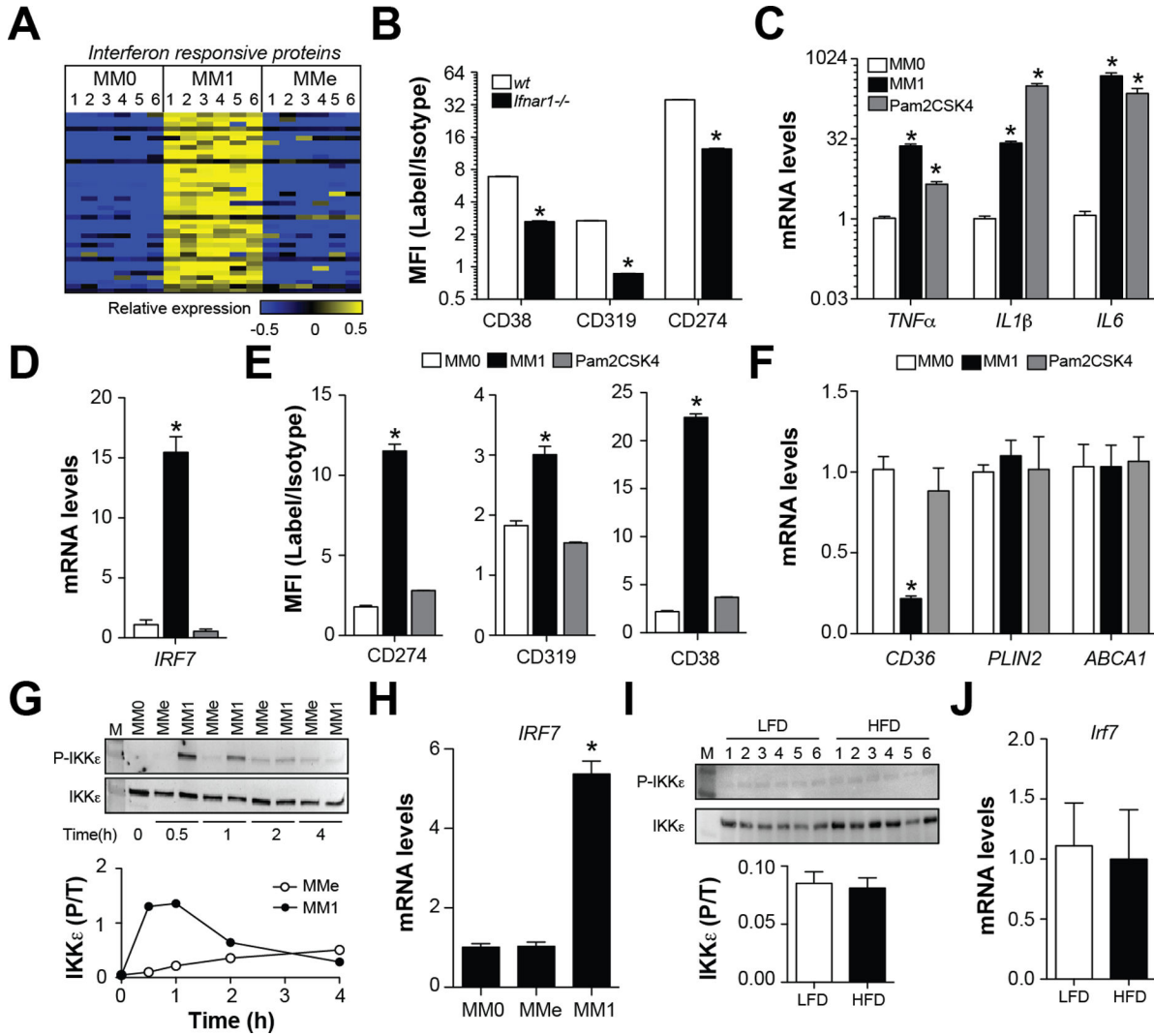


Fig. 4. ATMs display evidence of metabolic activation

Panel A: qRT-PCR analysis of human MM0 macrophages treated with media conditioned by human omental fat (Ad-CM) or sputum collected from a CF patient. *Inset:* Surface levels of CD38 and CD274. *Panel B:* qRT-PCR analysis of murine macrophages (MM0) treated with media conditioned by epididymal adipose tissue collected from obese (Ad-CM-HFD) or lean (Ad-CM-LFD) mice. *Panels C-E:* Omental (OM) and subcutaneous (SUBQ) adipose tissue was collected from obese subjects (BMI>30kg/m²) undergoing bariatric surgery (N=7). For comparative purposes, subcutaneous adipose tissue was collected from non-obese subjects (BMI<30kg/m²) undergoing abdominoplasty (N=7). *Panel C:* Cell surface levels of CD36 and ABCA1 in ATMs; results are signal-to-noise ratio in mean fluorescence intensity (MFI). *Panel D:* Relationships between BMI and cell surface CD36 or CD319 levels on ATMs (N=14). *Panel E:* Summary of linear regression analyses of cell surface protein levels on ATMs and BMI. *Panels F-G:* Male C57BL/6 mice were placed on a low-fat diet (LFD) or high-fat diet (HFD) for 16 weeks and ATMs from epididymal fat were purified and analyzed by qRT-PCR. Where applicable, results are means and SEMs; *, denotes $p < 0.05$, t -test relative to MM0 or non-obese or LFD; N=4–7. See also Fig. S3.



Human, Mouse

Fig. 5. Metabolic activation fails to induce the type I interferon response
Panel A: Heatmap depicts regulation of interferon-regulated proteins across human MM0, MM1, and MMe cells (blue = down-regulated, yellow = up-regulated). *Panel B:* Comparison of M1 cell surface marker levels in MM1 macrophages made from *wt* and *Ifnar*^{-/-} mice. *Panels C-F:* Human MM0 macrophages were classically activated or treated with Pam2CSK4, a selective agonist for TLR2. *Panel C:* Pro-inflammatory cytokine expression. *Panel D:* *IRF7* expression. *Panel E:* M1 cell surface markers were quantified by flow cytometry. *Panel F:* qRT-PCR analysis of MMe markers. *Panels G-H:* Human MM0 macrophages were classically or metabolically activated. *Panel G:* Immunoblotting for total and phosphorylated (P-Ser¹⁷²) IKK ϵ . *Panel H:* *IRF7* expression. *Panels I-J:* Male C57BL/6 mice were placed on a low-fat diet (LFD) or high-fat diet (HFD) for 16 weeks and ATMs from epididymal fat were isolated. *Panel I:* Immunoblotting for total and phosphorylated (P-Ser¹⁷²) IKK ϵ . *Panel J:* *Irf7* expression in purified ATMs. Where applicable, results are means and SEMs; *, denotes *p*<0.05, *t*-test relative to MM0; N=4–6. Flow cytometry results are expressed as signal-to-noise ratios in the mean fluorescence intensity (MFI). See also Fig. S5.

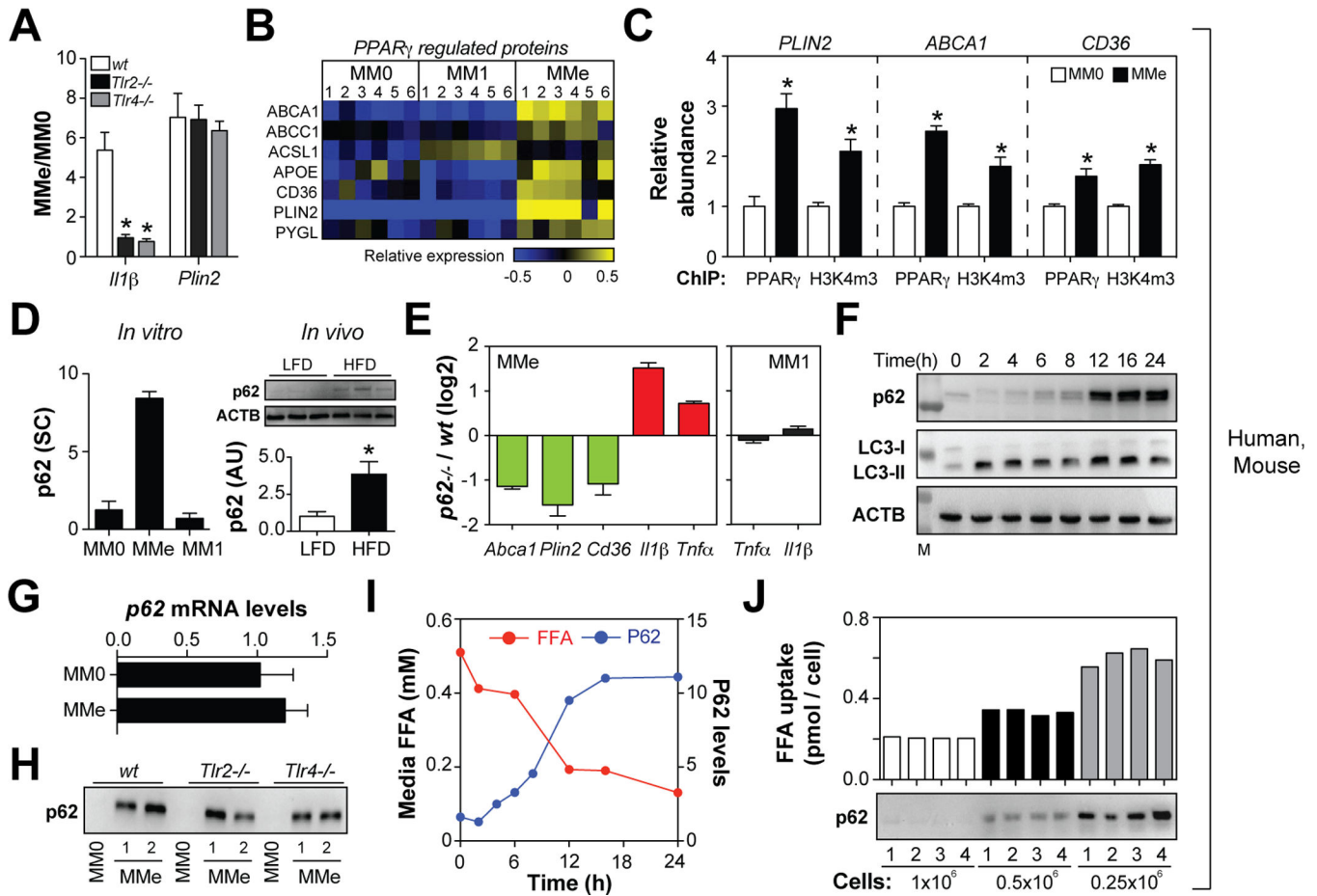


Fig. 6. p62 and PPAR γ promote MMe marker expression and limit inflammation

Panel A: Comparison of gene expression in MMe macrophages made from wild-type (*wt*), *Tlr2*^{-/-}, and *Tlr4*^{-/-} mice. *Panel B:* Heatmap depicts regulation of PPAR γ -regulated proteins across human MM0, MM1, and MMe cells (blue = down-regulated, yellow = upregulated). *Panel C:* PPAR γ binding to its target gene promoters is increased in MMe relative to MM0 macrophages, and correlated with abundance of the open chromatin mark H3K4m3. Relative abundance of MM0 ChIP levels were arbitrarily set to 1. *Panel D:* Protein levels of p62 in human macrophages *in vitro* (spectral counts, SC) and *in vivo* (arbitrary units, AU) in murine ATMs isolated from mice fed a low-fat (LFD) or high-fat diet (HFD). *Panel E:* Comparison of gene expression in MMe and MM1 macrophages from wild-type and *p62*^{-/-} mice. *Panel F:* Kinetics of p62 accumulation and LC3-I/-II levels in human MMe macrophages. *Panel G:* *p62* mRNA levels. *Panel H:* p62 levels in MM0 and MMe macrophages made from wild-type (*wt*), *Tlr2*^{-/-}, and *Tlr4*^{-/-} mice. *Panel I:* Kinetics of p62 accumulation and FFA uptake by human MMe macrophages. *Panel J:* Varying numbers of human macrophages were metabolically activated and palmitate uptake and p62 levels were determined. Where applicable, results are means and SEMs; *, denotes *p*<0.05, *t*-test relative to MM0 or LFD; N=3–6. See also Figs. S6, S7.

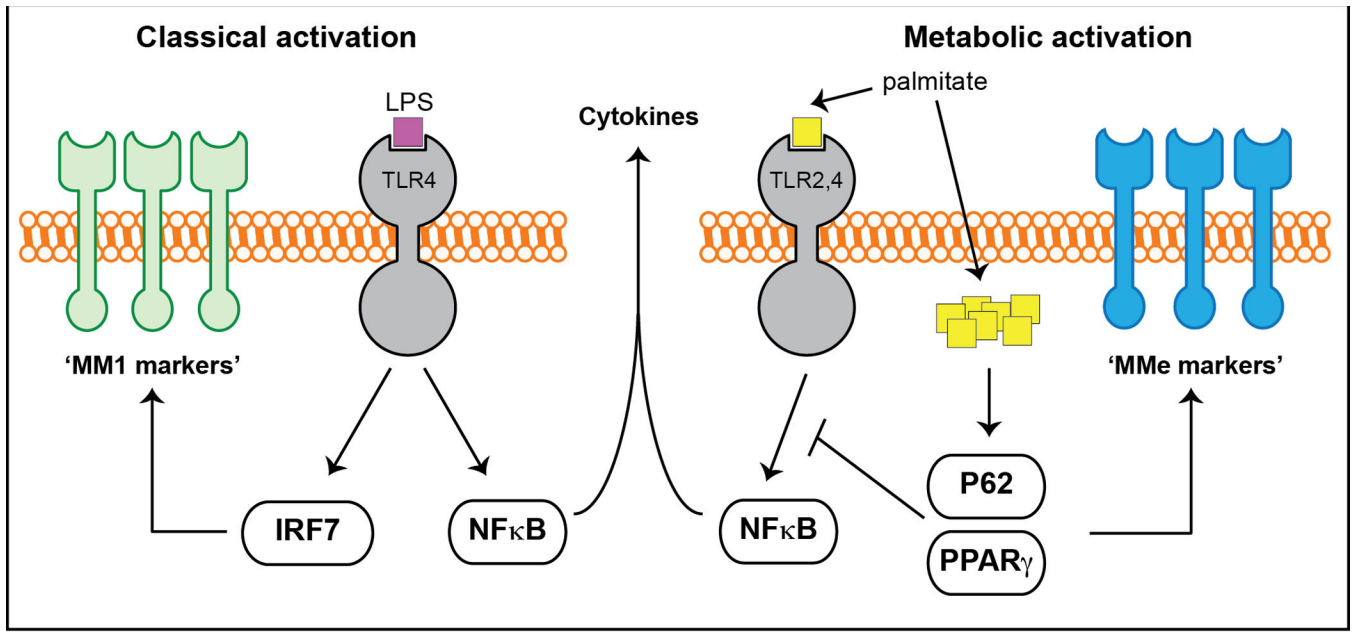


Fig. 7. A molecular model for metabolic and classical activation of macrophages
See text for details.